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Epidermal growth factor increases collagen production in granulation tissue by stimulation of fibroblast proliferation and not by activation of procollagen genes

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The effects of epidermal growth factor (EGF) on granulation-tissue formation and collagen-gene expression were studied in experimental sponge-induced granulomas in rats. After daily administration of 5 μg of EGF into the sponge, total RNA was extracted from the ingrown granulation tissue at days 4 and 7 and analysed by Northern hybridization for the contents of mRNAs for types I and III procollagens. EGF treatment increased procollagen mRNA, particularly at day 4. To determine whether this elevation was due to increased proliferation of collagen-producing fibroblasts or to activation of collagen-gene expression in these cells, fibroblast cultures were started from granulation tissue and treated with EGF. These experiments confirmed that EGF is a potent mitogen for granuloma fibroblasts in a dose-dependent manner. The effect of EGF treatment on radioactive hydroxyproline production in cultured cells was inhibitory. The decreased rate of collagen synthesis was also indicated by decreased amounts of procollagen mRNAs. The results suggest that the stimulation of wound healing and collagen production by EGF is due to increased fibroblast proliferation, and not to increased expression of type I and III procollagen genes.

INTRODUCTION

Epidermal growth factor (EGF) is considered to play a major role in induction of wound healing, along with the platelet-derived growth factor (PDGF) and the transforming growth factors α and β (TGF-α and TGF-β) (Sporn & Roberts, 1986). Treatment of experimental granulation tissue with EGF has been shown to result in increased contents of DNA, RNA and protein, particularly collagen, and of glycosaminoglycans (Niall *et al.*, 1982; Buckley *et al.*, 1985; Laato, 1986; Laato *et al.*, 1986*a*,*b*). The fact that the collagen content of experimental granulomas is increased dramatically after daily application or sustained release of EGF for 7 days (Laato *et al.*, 1986*a*; Buckley *et al.*, 1985) suggests that, in addition to its mitogenic activity, this growth factor could have a direct stimulatory effect on collagen-gene expression. Vicose-cellulose-sponge implants (Lampiaho & Kulonen, 1967) have previously been used to study the regulation of collagen biosynthesis in granulation tissue at the level of procollagen mRNAs (Mäkelä & Vuorio, 1986). In the present study a similar wound model was used to determine the effects of daily EGF administration on amounts of type I and III procollagen mRNA.

MATERIALS AND METHODS

Wound model

A standardized experimental wound model was used (Niinikoski *et al.*, 1971). Viscose-cellulose sponge was cut into cylindrical pieces with a tunnel in the centre, as described previously (Laato *et al.*, 1986*a*).

Experimental protocol

Altogether 24 rats were studied in four groups of six animals. In the control groups, the implants were treated immediately after implantation by injecting 0.05 ml of 0.1% (w/v) bovine serum albumin in phosphate-buffered saline (0.14 M-NaCl/0.01 M-sodium phosphate buffer, pH 7.3) into the central tunnel of the implant. The implants of the two test groups were injected correspondingly with 0.05 ml of a solution containing 5 μg of EGF in 0.1% bovine serum albumin. Recombinant human EGF was obtained as a gift from Chiron Corp. (Emeryville, CA, U.S.A.). The injections were repeated daily under strictly aseptic conditions. Bacteriological examinations of wound fluid were performed in each group at the end of experiments, and no infections were observed.

RNA extraction and fractionation

For collection of the granulomas at days 4 and 7, the rats were anaesthetized with diethyl ether and killed. After removal from killed animals, the implants were dissected free from surrounding tissues and the silicone discs were removed. The granulomas were cut into small slices, collected into liquid nitrogen and stored at -70 °C. Total RNA was extracted from pooled granulomas of two or three animals by the method of Rowe *et al.* (1978). For analyses the RNAs were denatured with glyoxal and dimethyl sulphoxide, fractionated on 0.75% agarose gels, and transferred to nitrocellulose by blotting (Thomas, 1980).

Cell-culture studies

Fibroblast cultures were started from rat experimental granulation tissue (Jalkanen, 1981). The cells were grown in Dulbecco's modification of Eagle's medium supplemented with 10% (v/v) fetal-calf serum. For passaging, the cells were detached with trypsin and divided 1:3. Treatment of cell cultures was performed by daily addition of EGF into the cell-culture medium, to final

Abbreviations used: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor.

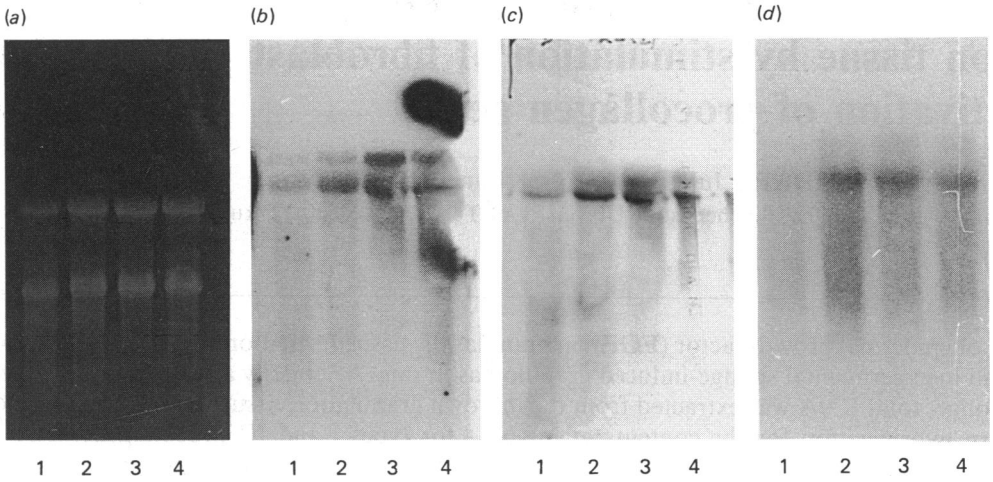

Fig. 1. Detection of type I and III procollagen mRNAs by hybridization

Total RNAs were isolated from EGF-treated and control granulomas at days 4 and 7. Samples (10 μg) of RNA were electrophoresed on 0.75%-agarose gels. One set of samples was stained with ethidium bromide to detect the rRNAs (*a*), and the others were transferred by blotting on to Pall Biodyne transfer membranes. These filters were hybridized with nick-translated [^{32}P]dCTP-labelled cDNA probes for rat proα1(I) collagen mRNA (*b*), rat proα2(I) collagen mRNA (*c*) and rat proα1(III) collagen mRNA (*d*). After washes, the hybridized probe was detected by exposure with X-ray films by using intensifying screens. The total RNA samples were: lane 1, control granuloma at day 4; lane 2, EGF-treated granuloma at day 4; lane 3, control granuloma at day 7; lane 4, EGF-treated granuloma at day 7.

concentrations of 1–100 μg/ml. The cells were harvested at days 4 and 7, and samples were counted in a Burker chamber. Cytoplasmic RNA was prepared from the cells and blotted on to nitrocellulose (White & Bancroft, 1982).

In some experiments the cultured granulation-tissue fibroblasts were also labelled with [^{3}H]proline in Dulbecco's medium without serum but supplemented with ascorbate (50 μg/ml) and β-aminopropionitrile (50 μg/ml), with or without EGF. After the labelling, the cells were detached, counted and processed as above. The culture media were analysed for the content of radioactive hydroxyproline by the method of Juva & Prockop (1966).

Hybridizations

The Northern- and slot-blot filters were analysed by using cDNA clones specific for rat proα1(I) collagen mRNA (pα1R2) and rat proα2(I) collagen mRNA (pα2R2) (Genovese *et al.*, 1984), and for rat proα1(III) collagen mRNA (pRGR5; J. K. Mäkelä & E. Vuorio, unpublished work). The plasmid DNAs were nick-translated to specific radioactivities of approx. 10^8 c.p.m./μg of DNA by using [^{32}P]dCTP. After prehybridization, the filters were hybridized with the probes and washed as described by Thomas (1980). The filters were exposed with X-ray films, and the extent of hybridization was quantified by densitometry of multiple exposures of the filters.

RESULTS

Procollagen mRNA contents in developing granulomas

To determine whether daily EGF administrations, known to increase the collagen content of the granulomas, act by altering the amounts of procollagen mRNAs, total RNA was extracted from experimental granulomas at days 4 and 7. Control RNA was obtained from similar implants receiving daily saline injections. Equal amounts of each total RNA preparation were analysed by Northern blotting (Fig. 1). EGF was found to cause a marked increase in mRNAs for proα1(I), proα2(I) and proα1(III) collagen in 4-day granulomas (Figs. 1 and 2). After 1 week's administration of EGF, the amounts of procollagen mRNAs, when calculated per total RNA, were essentially similar to those measured in control RNAs. If one takes into account the fact that EGF-treated granulomas had approx. 50% more total RNA per sponge (Laato *et al.*, 1986*a*), the amounts of procollagen mRNAs in each EGF-treated granuloma were at least 50% higher than in the control granulomas after treatment with EGF for 7 days.

Cell-culture studies

As the increased procollagen mRNA contents could result from increased transcription of the corresponding genes, or from an increase in the numbers of fibroblasts without changes in their collagen gene expression, or from a combination of the two, the effects of EGF on cultured rat granulation-tissue fibroblasts were also studied. First the dose–response curves of the cells to EGF were studied after 4-day incubations of subconfluent cells. The results in Fig. 3 show that EGF stimulated cell proliferation in a dose-dependent manner. Cytoplasmic slot-blot hybridizations were performed to measure mRNAs for type I and type III collagen. The results clearly show that EGF treatment did not cause increases in the cellular concentrations of mRNA for proα1(I) or proα1(III) collagen, but resulted in most cases in small decreases in these mRNAs, particularly when higher doses of EGF were used (Fig. 3). In further experiments EGF was used at a concentration of 10 ng/ml of culture medium. In all the experiments performed, EGF was found to stimulate proliferation of cultured granulation-

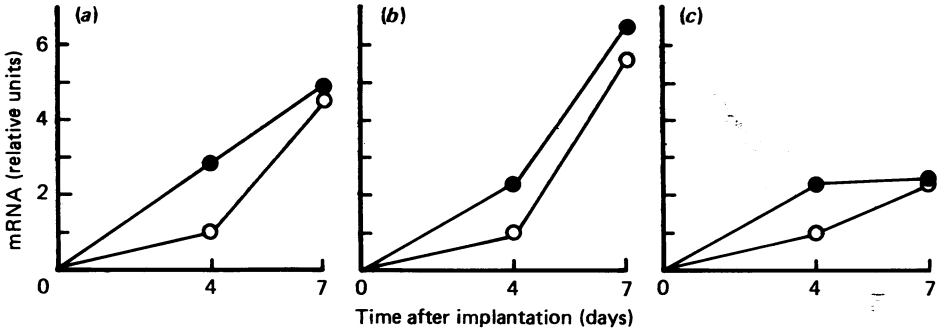

Fig. 2. EGF-induced changes in type I and III procollagen mRNA contents

Densitometric analyses of multiple exposures of X-ray films (as shown in Fig. 1) were used to measure the amounts of mRNAs for proα1(I) (*a*), proα2(I) (*b*) and proα1(III) collagen (*c*) relative to total RNA. The amounts observed for 4-day control samples were given the value of 1.0. ○, Control samples; ●, samples from EGF-treated granulomas.

tissue fibroblasts and to decrease their collagen synthesis (as determined by hydroxyproline radioactivity) and procollagen mRNA contents (Table 1).

DISCUSSION

The stimulatory role of EGF on granulation-tissue formation has been well established (Buckley *et al.*, 1985; Carpenter & Zendegui, 1986; Laato, 1986; Laato *et al.*, 1986*a*,*b*). In microscopic examination, sponges containing slow-release pellets of EGF stimulated tissue ingrowth and resulted in an enhanced organization of granulation tissue, and in greater angiogenesis than in the placebo-treated controls (Buckley *et al.*, 1985). Local application of an ointment containing EGF to rabbit ear wounds similarly increased the thickness and cellularity of epithelium, inhibited wound contracture, and enhanced connective-tissue maturation (Franklin & Lynch, 1979). In our previous studies (Laato, 1986; Laato *et al.*, 1986*a*,*b*), daily local application of 5 μg of EGF resulted in an augmented cellular response to injury, and biochemical analyses revealed dose-dependent increases in the accumulation of DNA, RNA, glycosaminoglycans and proteins, including collagen hydroxyproline (Laato *et al.*, 1986*a*,*b*). Furthermore, the daily EGF treatment increased the local blood flow of granulation tissue above the control value (Laato, 1986). Most of these observations can be explained by the mitogenic effect of EGF towards fibroblasts. However, the rate of collagen accumulation was also increased (Laato *et al.*, 1986*a*), and therefore the possibility of a direct activation of collagen gene expression had to be considered.

In the first experiments of the present study, RNA was extracted from experimental granulomas and analysed for the contents of type I and type III procollagen mRNAs. The hybridization experiments (Figs. 1 and 2) revealed increases in procollagen mRNAs, particularly after 4 days of EGF administration. This difference in the relative mRNA contents was greatly diminished by day 7. These findings suggest a greater rate of transcription of type I and type III collagen genes (or a specific mechanism increasing the half-lives of the corresponding mRNAs) in EGF-treated granulomas. The two potential explanations for this observation were: (1) individual fibroblasts have increased their contents of procollagen mRNAs or (2) the amount of fibroblasts in relation to other cell types during the early stages of granulation

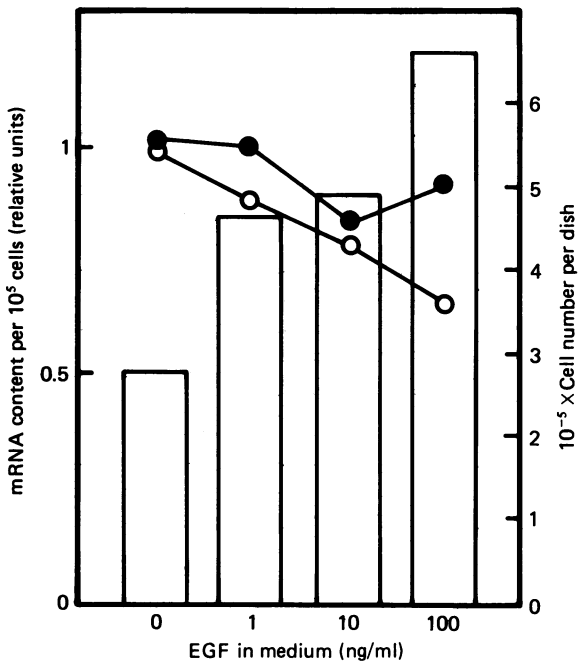

Fig. 3. Dose-dependent effects of EGF on fibroblast growth and procollagen mRNA amounts

Fibroblast cultures started from rat experimental granulation tissue were grown in 9 cm^2 Petri dishes as described in the text with or without EGF (1, 10 or 100 ng/ml) in the medium. The cells received daily EGF additions to the medium until they were harvested at day 4. Samples were taken for cell counting (shown as bars); the rest were processed for analysis of procollagen mRNA contents by cytoplasmic slot-blot hybridizations. The amounts of proα1(I) and proα1(III) collagen mRNAs are shown as ○ and ●, respectively, in densitometric units per 10^5 cells. All analyses are based on triplicate samples.

tissue formation has greatly increased. An increase in the relative number of collagen-producing cells could thus result in an increase in the amount of procollagen mRNAs relative to total RNA in the tissue.

To study these two possibilities, fibroblast cultures started from rat experimental granulomas were treated with EGF. In all the experiments, the stimulatory effect

Table 1. Effects of EGF treatment on cultured granuloma fibroblasts

Experimental granuloma-derived fibroblasts in cell culture were treated daily with 10 ng of EGF/ml in the culture medium for 4 days. At the end of the incubation, the cells were detached with trypsin. Samples were counted in a Burker chamber for estimation of cell numbers. The rate of collagen synthesis was studied in parallel cultures by labelling the cultures for 24 h with [^{3}H]proline, followed by determination of radioactive hydroxyproline as described in the text. Cytoplasmic concentrations of proα1(I) and proα1(III) collagen mRNAs were estimated by hybridization of the slot blots.

Cultures ...	Control	EGF-treated
10^{-6} × Cell number*	28.6±0.1 (4)	51.1±0.1 (4)
Radioactive hydroxyproline (d.p.m./cell)*	1953±71 (4)	941±39 (4)
Proα1(I) collagen mRNA†	1.0 (0.69–1.31) (6)	0.78 (0.51–0.91) (6)
Proα1(III) collagen mRNA†	1.0 (0.79–1.20) (6)	0.82 (0.62–1.0) (7)

* Expressed as means±S.E.M. for four cultures.
† Expressed as relative densitometric units (and range). The number of cell cultures studied is given in parentheses.

of EGF on fibroblast proliferation was clearly established. In the same cultures the rate of radioactive hydroxyproline production was, however, decreased to approximately half of that seen in untreated controls. Cytoplasmic slot hybridizations revealed that procollagen mRNA contents were also somewhat decreased in these cells, although the total amount of these mRNAs (per cell culture) was increased, owing to a 2-fold increase in the cell number. Although the cell-culture experiments cannot be directly compared with those carried out *in vivo*, we interpret the data to indicate that EGF does not induce elevated procollagen-mRNA contents in the granulation-tissue fibroblasts. In another cell-culture study the effects of EGF on collagen production were found to be negligible (Roberts *et al.*, 1986). The decrease in collagen synthesis and in procollagen mRNA in the EGF-treated groups may reflect the observations that rapidly proliferating fibroblasts have decreased contents of procollagen mRNAs (Miskulin *et al.*, 1986).

The EGF-induced increased rate of wound healing, whether detected by histology and morphometry, by DNA, RNA or collagen determinations, or by mRNA determinations, appears to result from the elevated rate of proliferation of granulation-tissue fibroblasts. Although EGF treatment greatly increased the amounts of type I and type III procollagen mRNAs in 4-day granulomas, the amounts observed in 7-day granulomas were nearly the same in EGF-treated and control tissues. These findings suggest that EGF treatment enhances wound healing by increasing fibroblast proliferation, but does not result in collagen overproduction. Fibrosis could be a major side effect of growth factors which stimulate collagen-gene expression. Lack of such an activity in EGF makes this growth factor a potentially useful therapeutic agent.

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